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RESEARCH PAPER

Selected ginsenosides of the protopanaxdiol series are novel positive allosteric modulators of P2X7 receptors

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BACKGROUND AND PURPOSE

The P2X7 receptor is an ATP-gated ion channel predominantly expressed in immune cells and plays a key role in inflammatory processes. Ginseng is a well-known Chinese herb with both pro- and anti-inflammatory properties and many of its actions have been ascribed to constituent ginsenosides. We screened a number of ginsenoside compounds for pharmacological activity at P2X7 receptors, that might contribute to the reported immunomodulatory actions of ginseng.

EXPERIMENTAL APPROACH

We used several assays to measure responses of P2X7 receptors, ATP-mediated dye uptake, intracellular calcium measurement and whole-cell patch-clamp recordings. HEK-293 cells stably expressing human P2X7 receptors were used in addition to mouse macrophages endogenously expressing P2X7 receptors.

KEY RESULTS

Four ginsenosides of the protopanaxdiol series, Rb1, Rh2, Rd and the metabolite compound K (CK) potentiated the dye uptake responses of P2X7 receptors, whereas other ginsenosides tested were ineffective (1–10 μM). The potentiation was rapid in onset, required a threshold concentration of ATP (>50 μM) and had an EC_{50} of 1.08 μM. CK markedly enhanced ATP-activated P2X7 currents, probably via an extracellular site of action. One of the consequences of this potentiation effect is a sustained rise in intracellular Ca^{2+} that could account for the decrease in cell viability in mouse macrophages after a combination of 500 μM ATP and 10 μM CK that are non-toxic when applied alone.

CONCLUSIONS AND IMPLICATIONS

This study identifies selected ginsenosides as novel potent allosteric modulators of P2X7 channels that may account for some of the reported immune modulatory actions of protopanaxdiol ginsenosides *in vivo*.

Abbreviations

AM, acetoxymethyl; CK, compound K; HEK-hP2X7, HEK-293 cells stably expressing human P2X7 receptors; PPD, protopanaxdiol; PPT, protopanaxtriol

Tables of Links

TARGETS
Ligand-gated ion channels[a]
GABAA receptors
P2X7 receptors
Ion channels[b]
HERG (Kv11.1) channels

LIGANDS
A-438079
ATP
BzATP
Clemastine
Rg3 ginsenoside

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013a,b).

Introduction

Ginseng has been used for at least 2000 years in China and other Asian countries to support vitality and long life. In traditional Chinese medicine, it is often described as a 'precious tonic' that stimulates natural resistance to infection and maintains homeostasis (Hanley *et al.*, 2012; Kumagai *et al.*, 2013). It is typically extracted from the roots of *Panax ginseng* C Mayer (Asian) and *Panax quinquefolius* (American) and contains a complex mixture of bioactive compounds; the most extensively studied being the 'steroid-like' dammarane triterpenoid glycosides, termed ginsenosides. Ginsenosides are further subdivided into protopanaxdiols (PPD) (examples shown in Figure 1) or protopanaxtriols (PPT) depending on the location of the sugar moieties on the dammarane carbon skeleton. Both glycosylated classes may have sugar moieties on carbon (C) 20 but PPD can have additional sugar moieties on C-3 whereas PPT are on C-6. The aglycones of both classes differ only in the presence of an extra hydroxyl group on C-6 in the triol (Tomasinsig *et al.*, 2008; Chotjumlong *et al.*, 2013). Ginseng is commercially available in a variety of forms (capsules, tablets, oils) where the type and amounts of constituent ginsenosides are precisely controlled. The G115 formulation tested in this study contains 4% w/w ginsenosides and is a principal constituent of Ginsana® and Gincosan® medications (Flordis, St Leonards, NSW, Australia).

We are interested in understanding the mechanisms underlying the reported immunomodulatory effects of ginseng/ginsenosides as they may translate into effective medications for the prevention and treatment of infective and inflammatory diseases. *In vivo* studies using ginseng formulations have been shown to protect against lung infections caused by *Pseudomonas aeruginosa* improving pulmonary bacterial clearance (Song *et al.*, 1997a,b; 2010) and a clinical trial demonstrated a significant reduction in the number of cases of influenza when given as an adjuvant with vaccine that correlated with increased antibody titres and NK cell activity (Scaglione *et al.*, 1996). Similar adjuvant effects have also been consistently demonstrated with ginsenosides *in vivo* (Rivera *et al.*, 2005; Han and Rhew, 2013). *In vitro*, ginsenosides have been shown to either trigger or prevent apoptosis depending on cell type or the specific ginsenoside used (Ham *et al.*, 2006; Zhang *et al.*, 2008; 2013; Li *et al.*, 2012; Zheng *et al.*, 2014). A number of studies have also demonstrated that ginsenosides can act via either genomic Eβ or glucocorticoid receptors in the submicromolar range to differentially regulate angiogenesis in endothelial cells (Sengupta *et al.*, 2004; Leung *et al.*, 2006; 2007; 2009). However, similar to some endogenous steroids, ginsenosides have also been shown to rapidly and reversibly interact with ligand-gated ion channels which points to additional non-genomic sites of action (Nah, 2014). These include both inhibitory actions on nicotinic ACh receptors (Lee *et al.*, 2003), 5-HT_3 receptors (Choi *et al.*, 2003a), NMDA receptors (Kim *et al.*, 2002), $GABA_A$ receptors (Lee *et al.*, 2012), TRPV1 channels (Huang *et al.*, 2012) and potentiating actions on glycine (Noh *et al.*, 2003), $GABA_A$ receptors (Choi *et al.*, 2003b) and TRPV1 channels (Jung *et al.*, 2001).

In this study, we tested the effects of ginsenosides on a subtype of ATP-gated ion channels of the P2X family, P2X7 receptors, given their important role in regulating immune cell function (Bartlett *et al.*, 2014). Studies have demonstrated P2X7 receptors to play a role in Ca^{2+} signalling, reactive oxygen species generation and cell death pathways and regulation of such signalling pathways may control immune responses (Bartlett *et al.*, 2014). We report that several glycosylated PPD ginsenosides, but not PPT ginsenosides, appear to be positive allosteric modulators of the ATP-activated P2X7 channel which can lead to enhanced Ca^{2+} influx and subsequent apoptosis in macrophages. Due to the relatively high expression levels of P2X7 receptors in immune cells, this action may account for some of the reported immune modulatory actions of PPD ginsenosides *in vivo*.

Methods

Cell culture

HEK-293 cells stably transfected with the human P2X7 receptors (HEK-hP2X7) plasmid (clone pJB3) were maintained in DMEM : F12 media (Life Technologies catalogue number 11320-033) supplemented with 10% FBS (French origin, Bovogen Biologicals, Keilor East, Victoria, Australia), 1% Glutamax, 10 000 U·mL^{-1} penicillin and 10 mg·mL^{-1} streptomycin with selection under 400 μg·mL^{-1} G418 (Life Technologies). The J774 murine macrophage cell line was maintained in RPMI 1640 media supplemented with 10% FBS, 1% Glutamax and 1% penicillin and streptomycin (Bhaskaracharya *et al.*, 2014).

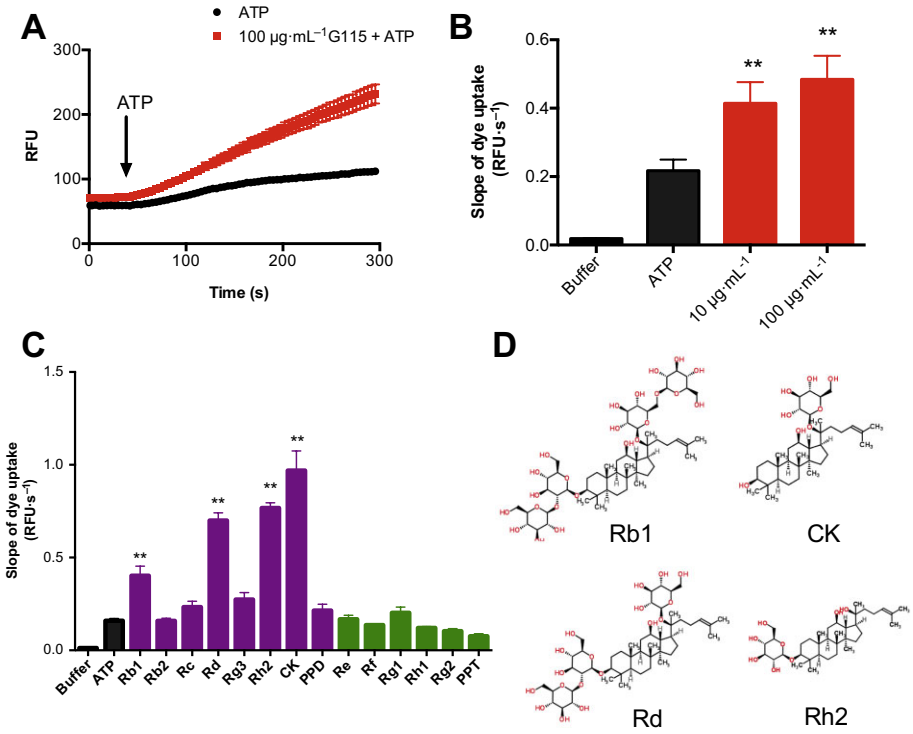

Figure 1

G115 ginseng formulation and purified ginsenosides potentiate ATP-induced responses at the human P2X7 receptor. (A) ATP-induced dye uptake was measured at 37°C using YOPRO-1 (2 µM) as the membrane impermeant dye. Relative fluorescence units (RFU) were measured following excitation at 490 nm and emission recorded at 520 nm using a fluorescent plate reader (Flexstation III). HEK-hP2X7 cells were pretreated with 10 or 100 µg·mL^{-1} G115 in a low divalent buffer for 10 min at 37°C. ATP (200 µM) was then added to elicit a P2X7 receptor response. The mean of five individual wells is plotted. (B) Mean slope data (n = 10–20 wells) is plotted for buffer control, ATP or ATP in the presence of 10 or 100 µg·mL^{-1} G115. Error bars are SEM. **denotes $P < 0.001$ using ANOVA with Dunnett's multiple comparison test. (C) A total of 14 purified ginsenoside compounds were tested for potentiation of P2X7 receptor responses at a concentration of 10 µM. All compounds were prepared in DMSO and were added to the buffer with low concentrations of divalent cations. Compounds were pre-incubated for 10 min prior to the addition of ATP (200 µM). (D) Chemical structures of ginsenosides with potentiating effect on human P2X7 receptors.

Animals

All animal care and experimental procedures complied with Australian guidelines for the use of animals and were approved by the local RMIT Animal Ethics Committee (approval number AEC1312). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 3 animals were used in the experiments described here.

Adult male C57BL/6 mice (8–12 weeks of age, from Animal Resources Centre, Perth, Australia) were maintained in a 12 h light/dark cycle and fed with standard diet with water *ad libitum*. Mice were killed by CO_2 asphyxiation and peritoneal macrophages were obtained by flushing the peritoneal cavity with cold PBS (5 mL).

Dye uptake experiments

Cells were plated at 2.5 × 10^4 cells per well the day before experiments into poly-D-lysine coated 96-well plates (ThermoFisher Scientific Australia, Scoresby, Victoria, Australia). YOPRO-1 iodide (Life Technologies, Mulgrave, Australia) was used as the membrane impermeant dye with a final concentration of 2 µM in low divalent buffer (145 mM NaCl, 5 mM KCl, 0.2 mM $CaCl_2$, 13 mM glucose, 10 mM HEPES, pH 7.3, osm 300–310). A Flexstation III plate reader (Molecular Devices, Sunnyvale, CA, USA) was used to acquire data using the following settings – excitation wavelength 490 nm, emission wavelength 520 nm and six reads per well. Data were analysed as slope of dye uptake or AUC between 40 and 300 s using SoftMax Pro software (Molecular Devices).

Calcium measurements

HEK-293 cells were plated at 2.5 × 10^4 cells per well the day before experiments into poly-D-lysine coated 96-well plates. Cells were loaded with 1 µM Fluo-4-acetoxymethyl (AM) calcium indicator dye in low divalent buffer for 30 min at 37°C. This solution was then removed and replaced with standard extracellular assay buffer (145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 13 mM glucose, 10 mM HEPES, pH 7.3). A Flexstation III plate reader was used to acquire data using the following settings – excitation wavelength 490 nm, emission wavelength 520 nm and six reads per well. ATP was injected automatically after 40 s.

J774 mouse macrophages were plated at 2.5×10^4 cells per well into poly-D-lysine coated 96-well plates. Cells were loaded with 2.5 μM Fura-2AM calcium indicator dye plus an equal volume of pluronic acid in HBSS buffer for a total of 30 min at 37°C. This loading solution was removed and replaced with standard extracellular assay buffer. A Flexstation III plate reader was used to acquire data using the following settings: excitation wavelengths 340 nm and 380 nm with an emission wavelength of 520 nm. Six reads per well was used and ATP (10× concentration) was injected automatically after 40 s.

Peritoneal macrophages were plated onto 12 mm glass coverslips at 10 000 cells per slip and cultured overnight. Macrophages were loaded with 1 μM Fluo-4AM in low divalent buffer for 30 min at 37°C and then placed into a heated (36°C) 35 mm bath chamber on a Nikon Eclipse Ti-U fluorescent microscope (Coherent Scientific, Hilton, SA, Australia). Cells were continuously perfused with low divalent assay buffer by gravity feed and calcium signals measured using a Photometrics CoolSnap HQ2 CCD camera (Coherent Scientific) recording green fluorescence at 520 nm. A time-lapse recording was generated with exposure time of 300 ms and gain of 500 controlled through NIS Elements software (Nikon Instruments, Coherent Scientific). Regions of interest were drawn around 150–170 individual cells to measure fluorescence at each time point, background subtracted and the mean fluorescence value calculated.

Patch-clamp electrophysiology

Stably expressing HEK-hP2X7 cells were plated onto 13 mm glass coverslips 4–24 h before use. Membrane currents were recorded in the whole-cell patch-clamp configuration using an EPC10 amplifier (HEKA, Lambrecht, Germany) and borosilicate glass electrodes (Clark Electromedical, SDR Clinical Technology, Sydney, NSW, Australia), resistance 3–8 Ω when filled with standard internal solution. Cells were continually perfused by gravity feed with standard divalent buffer solution (145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 13 mM glucose, 10 mM HEPES, pH 7.3) prior to seal formation and with low divalent buffer solution in the majority of the experiments. Standard internal buffer solution contained NaCl 145 mM, HEPES 10 mM, EGTA 10 mM, pH 7.3 with NaOH 10 M. In certain experiments, investigating permeability changes of P2X7 channels, all cations in the external solution were substituted with the large organic cation N-methyl-D-glucamine (NMDG). This solution contained NMDG 154 mM, HEPES 10 mM, Glucose 13 mM, pH 7.3 with HCl 10M. Series resistance compensation was routinely applied up to a maximum of 80% to minimize voltage errors. ATP and drugs were applied using a computer-controlled fast-flow system (Bio-Logic Instruments, Claix, France) with the perfusion capillaries placed in close proximity to the cell under investigation.

Cell viability assays

Cells were plated at a density of 5×10^4 cells per well (50 μL) in a 96-well plate (Costar ThermoFisher Scientific) in triplicate. Compounds (or vehicle control) were added to the plate at two times final concentration and cells were treated for 24 h. Cell viability was assessed using the CellTiter Glo Aqueous One solution (Promega, Madison, WI, USA) which was added to the wells for the last 4 h of the experiment. Absorbance was read at 490 nm using a Clariostar plate reader (BMG Labtech Australia, Mornington, Victoria, Australia).

Data analysis

Graphs were plotted using GraphPad Prism version 6 (La Jolla, CA, USA). Concentration-response curves were fitted using a log (agonist) versus response – variable slope (four parameter) best-fit equation. Data were analysed for statistical significance using either unpaired *t*-tests or one-way ANOVA with post-tests as appropriate. Significance was taken as $P < 0.05$.

Materials

The P2X7 receptor agonists ATP and BzATP were obtained from Sigma Aldrich (Ryde, NSW, Australia). The P2X7 receptor antagonists AZ-10606120 and A-438079 hydrochloride were obtained from Tocris Biosciences (Bristol, UK). ATP was prepared as a 100 mM stock in double distilled water, pH to 7.4 with NaOH and was stored at –80°C until the day of experiment. AZ-10606120 and A-438079 (10 mM in DMSO) were stored at –20°C. Ginsenosides (certified as 98 % pure) were obtained from Chengdu Mansite Pharmaceutical Co Ltd (PPT, Rg1, Rg3, Rb1, Rb2, CK), Sichuan Weikeqi Biological Technology Co. Ltd (PPD, Rf), Chengdu Biopurify Phytochemicals Ltd (Rc, Rd, Re) and Shanghai E Star Bio Technology Co Ltd (Rh1, Rh2 (all supplied from Biopurify Phytochemicals Ltd, China)). Each compound was prepared as a 50 mM stock in DMSO and stored at –80°C until the day of the experiment. All ginsenoside stocks were further diluted in DMSO to 1000× final concentration so that when diluted in assay buffers, the final concentration of DMSO was 0.1%.

Results

Ginsenosides increase the rate of dye uptake through activated P2X7 receptors

To establish any potential effects of ginseng on P2X7 receptors, we used a standard screening assay that relies upon the uptake of the membrane impermeant dye YOPRO-1 iodide through the P2X7-dependent permeability pathway activated with the agonist ATP (Jursik *et al.*, 2007; Bhaskaracharya *et al.*, 2014). The initial screening of compounds used an approximate EC_{50} concentration of ATP (200 μM) in order to see either potentiation or inhibition of the response. We first tested a standard formulation of ginseng known as G115 as it is one of the main formulations used in the clinic. Pretreatment of HEK-hP2X7 cells for 10 min with 100 $\mu g \cdot mL^{-1}$ G115 enhanced the rate of ATP-induced dye uptake by around twofold (Figure 1A, B). G115 alone did not induce dye uptake in HEK-hP2X7 cells (Supporting Information Fig. S1), suggesting this was not because of a non-specific effect on the cells.

G115 contains a fixed amount (4% w/w) of eight ginsenosides from both PPD and PPT chemical classes, namely Rb1, Rc, Rd, Re (PPD ginsenosides) and Rb2, Rf, Rg1, Rg2 (PPT ginsenosides). It was important to establish whether the observed effects with G115 resulted from one or more specific ginsenoside(s) in the formulation. We tested 14 purified ginsenosides in a screening assay encompassing the eight ginse-

nosides in G115 plus the principal intestinal metabolites, Rh1, Rh2, Rg3, compound K (CK), and the two aglycones, PPD and PPT. All ginsenosides were tested at a concentration of 10 μM and were added 10 min prior to the addition of ATP (200 μM). None of the ginsenosides directly stimulated dye uptake (Supporting Information Fig. S1), however, four PPD ginsenosides, Rb1, Rd, Rh2 and CK, significantly increased the rate of dye uptake after ATP addition (Figure 1C). In contrast, ginsenosides of the PPT series (Figure 1C) had no significant effect on the ATP-induced dye uptake. The chemical structures of the four ginsenosides with effect on P2X7 receptors are shown in Figure 1D.

A glucopyranoside sugar residue in CK is essential for rapid potentiation of P2X7 receptors

Because the PPD aglycone was ineffective in potentiating responses of P2X7 receptors at concentrations up to 50 μM (data not shown), there is an absolute requirement for at least one sugar residue in this structure. Based on our data, this could be located at C-3 (Rh2) or C-20 (CK). As CK appeared to be the most potent in this assay and is one of the principal metabolites of ginseng, reaching plasma concentrations of around 70 ng·mL^{-1} in humans (Kim *et al.*, 2013), we focused our investigation on this ginsenoside to examine the potentiating action on P2X7 receptors in more detail.

The potentiation of ATP responses at P2X7 receptors by CK was also demonstrated in buffer containing physiological divalent ion concentrations (2 mM Ca^{2+}, 1 mM Mg^{2+}). Under these conditions much higher concentrations of ATP (>500 μM) are required to elicit any significant dye uptake, most likely because of the actions of these divalent ions in blocking responses of P2X7 receptors (Virginio *et al.*, 1997). CK could potentiate both 0.5 mM and 3 mM ATP responses (Figure 2A). The onset of potentiation of P2X7 receptor responses by CK was rapid with immediate effects observed following co-injection of a premixed cocktail of the agonist ATP and CK (Figure 2B). The response induced by ATP and CK was solely dependent on P2X7 receptors as it could be completely abolished by pretreatment with either of two selective P2X7 receptor antagonists; AZ10606120 (10 μM) and A-438079 (10 μM) (Figure 2C).

Our initial experiments suggested that CK was not a direct activator or agonist of P2X7 receptors as CK could not directly stimulate dye uptake in HEK-hP2X7 cells (Supporting Information Fig. S1). The EC_{50} value of the potentiation effect of CK on hP2X7 receptors was calculated as 1.08 μM (95% CI 0.86 to 1.35 μM) (Figure 3A). In contrast, the aglycone PPD was ineffective at all concentrations tested (0.1–50 μM). To determine if CK was acting as a positive allosteric modulator of P2X7 receptors, we performed a full concentration-response curve for ATP in the absence and presence of 10 μM CK (Figure 3B). Potentiation by CK left-shifted the concentration-response curve reducing the EC_{50} from 244 to 71 μM, increased the maximum ATP response and required a threshold concentration of ATP (~50 μM). In contrast, when BzATP was used as a full agonist at human P2X7 receptors (Surprenant *et al.*, 1996), CK did not increase the maximum response but did decrease EC_{50} from 19 to 4.4 μM (Figure 3B). Both leftward shifts were statistically significant ($P < 0.01$).

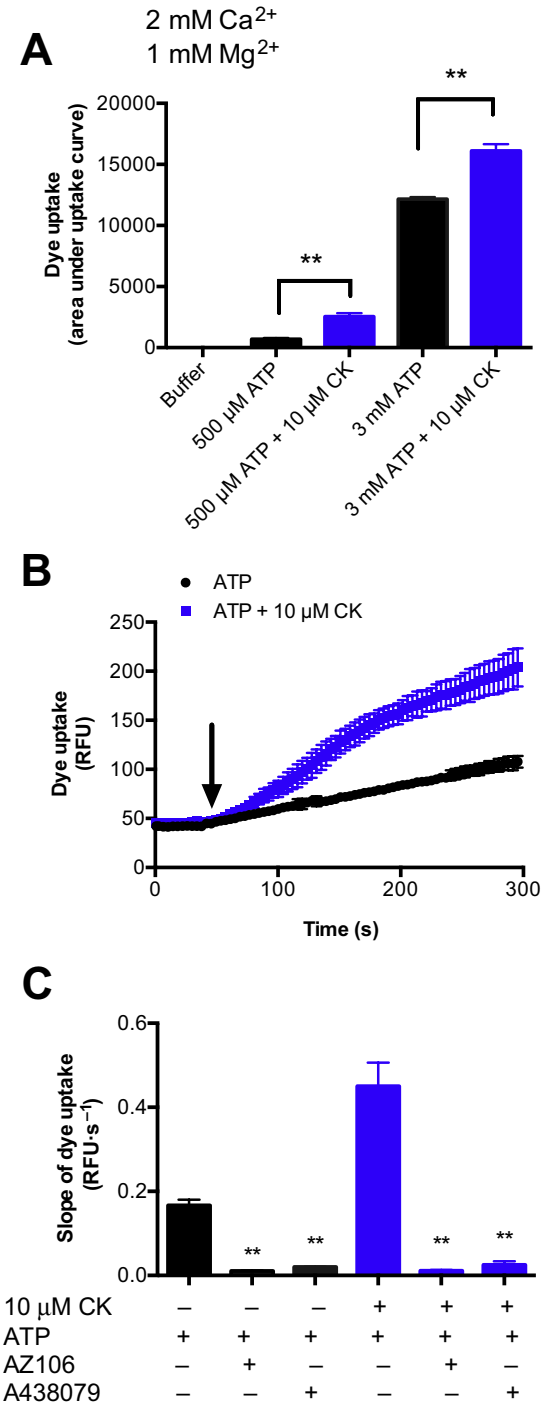

Figure 2

Ginsenoside CK acts rapidly to potentiate P2X7 receptor responses and is prevented by selective antagonists. (A) CK can potentiate P2X7 receptor responses in buffer containing physiological concentrations of $CaCl_2$ and $MgCl_2$. Data are collated from 5 to 9 individual wells. $^{**}P < 0.05$, significantly different as indicated; one-way ANOVA with Dunnett's post-test. (B) Dye uptake plot showing co-injection of 100 μM ATP plus 10 μM CK compared with 100 μM ATP alone. The mean ± SEM response from three individual wells is shown. (C) Mean data from 7 to 8 wells measuring ATP-induced dye uptake. Selective antagonists AZ10606120 and A-438079 inhibited the response. $^{**}P < 0.05$, significantly different to ATP control; one-way ANOVA.

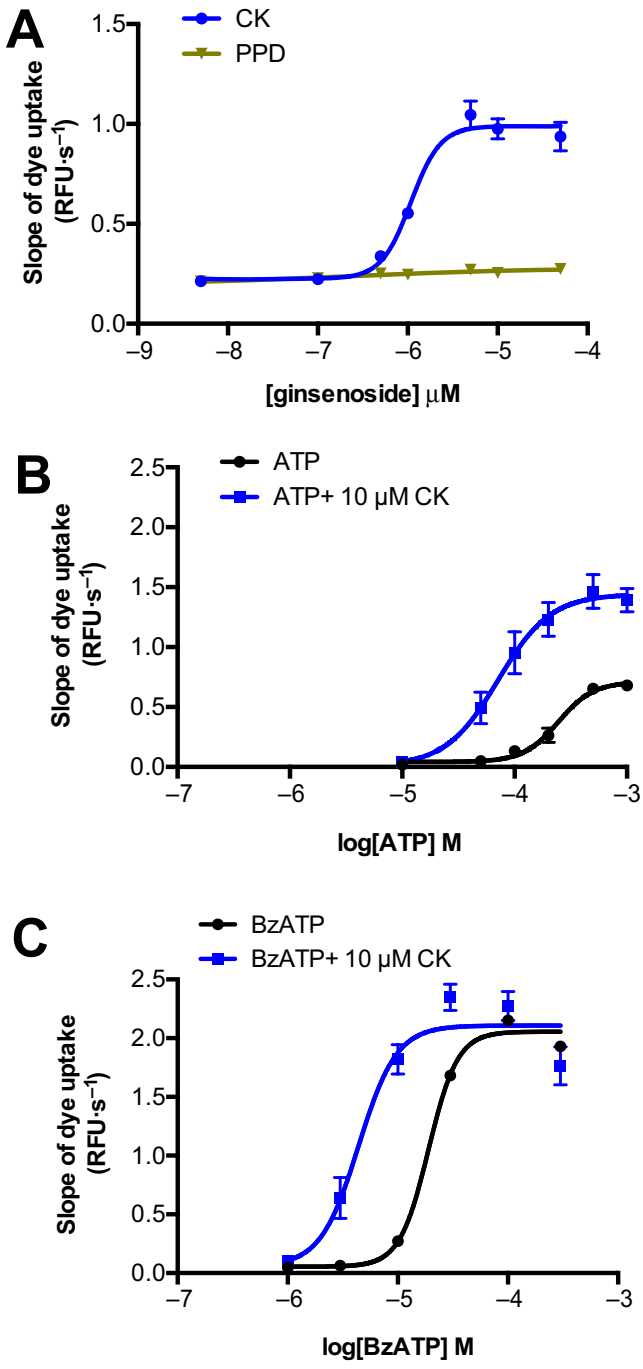

Figure 3

CK acts as a positive allosteric modulator of P2X7 receptors. (A) A concentration-response curve for the potentiating effect of CK. PPD is included as a control. (B) Concentration-response curves were generated for ATP (10 μM – 1 mM), with or without 10 μM CK. Data are the means, with SEM, of five independent experiments. (C) Concentration-response curves were generated for BzATP (1 μM – 0.3 mM), with or without 10 μM CK. Sigmoidal dose responses were fitted in GraphPad Prism.

Ginsenoside CK increases sustained calcium influx through P2X7 receptors

Due to their high permeability to Ca^{2+}, activation of P2X7 receptors can result in a sustained rise in intracellular Ca^{2+} leading to either proliferation/activation or cell death, depending on the magnitude and extent of channel activation (Adinolfi *et al.*, 2005b). Therefore, it was important to establish whether the ginsenoside potentiation of P2X7 receptor responses observed in dye uptake experiments lead to physiologically relevant sustained increases in intracellular Ca^{2+} concentration. We first investigated differences in intracellular Ca^{2+} responses between HEK-hP2X7 cells and the parental HEK-293 cell line (Figure 4A) using fluo-4AM loaded cells. In both cell lines there was an initial transient rise in intracellular Ca^{2+} following ATP addition which is due to the activation of G-protein coupled P2Y receptors. However, only HEK-hP2X7 cells showed an additional sustained elevation in intracellular Ca^{2+} on addition of ATP concentrations > 100 μM (Figure 4A). In the presence of ginsenoside CK, this sustained calcium response was observed with 100 μM ATP (Figure 4B, blue trace). Quantification of the changes in fluo-4 relative fluorescence units (both peak response and sustained response) show that CK significantly increases the ATP-mediated rise in intracellular Ca^{2+} only in P2X7 receptor-expressing cells (Figure 4C).

Ginsenosides are potent modulators of ATP-evoked currents

To definitively demonstrate that PPD ginsenosides enhanced ionic flux through activated P2X7 channels, we used the whole-cell patch-clamp technique and delivery of ATP, with or without ginsenosides, using an eight-channel fast-flow system. Our standard patch protocol used an initial application of 200 μM ATP for 5 s then switching to 200 μM ATP plus ginsenoside for 5 s before briefly returning to ATP alone (2 s) prior to wash off (Figure 5A–D). Representative traces for CK, Rd, Rb1, PPD, PPT, Rg1 and Rh1 are shown. As in the dye uptake experiments, we observed that only the glycosylated PPD ginsenosides potentiated ATP responses, whereas the PPD aglycone and all PPT ginsenosides had no effect at 50 μM, the highest concentration tested. The rank order of potency was CK > Rd >Rb1 with thresholds for effect around 50 nM, 0.5 μM and 10 μM respectively. These effects were rapid in onset occurring in <1 s of switching from ATP alone to ATP plus ginsenoside. Similarly the effects were rapidly reversible on wash-out of the ginsenoside in the continued presence of ATP. Consistent with the dye uptake data, the most potent ginsenoside CK did not directly activate P2X7 channels but potentiated current only after prior activation with ATP (data not shown). The magnitude of potentiation was quantified by dividing the current amplitude after 5 s in ATP + ginsenoside by the amplitude in ATP alone immediately prior to ginsenoside addition (Figure 5E). Although all the PPD ginsenosides had a dose-dependent effect in regard to potentiating ATP-evoked currents, it was not possible to demonstrate a maximal effect where further increases in ginsenoside concentration lead to no further increases in the potentiation. This was due to the fact that at higher concentrations of PPD ginsenosides, the potentiating effect was so large that the cells could not be effectively voltage clamped.

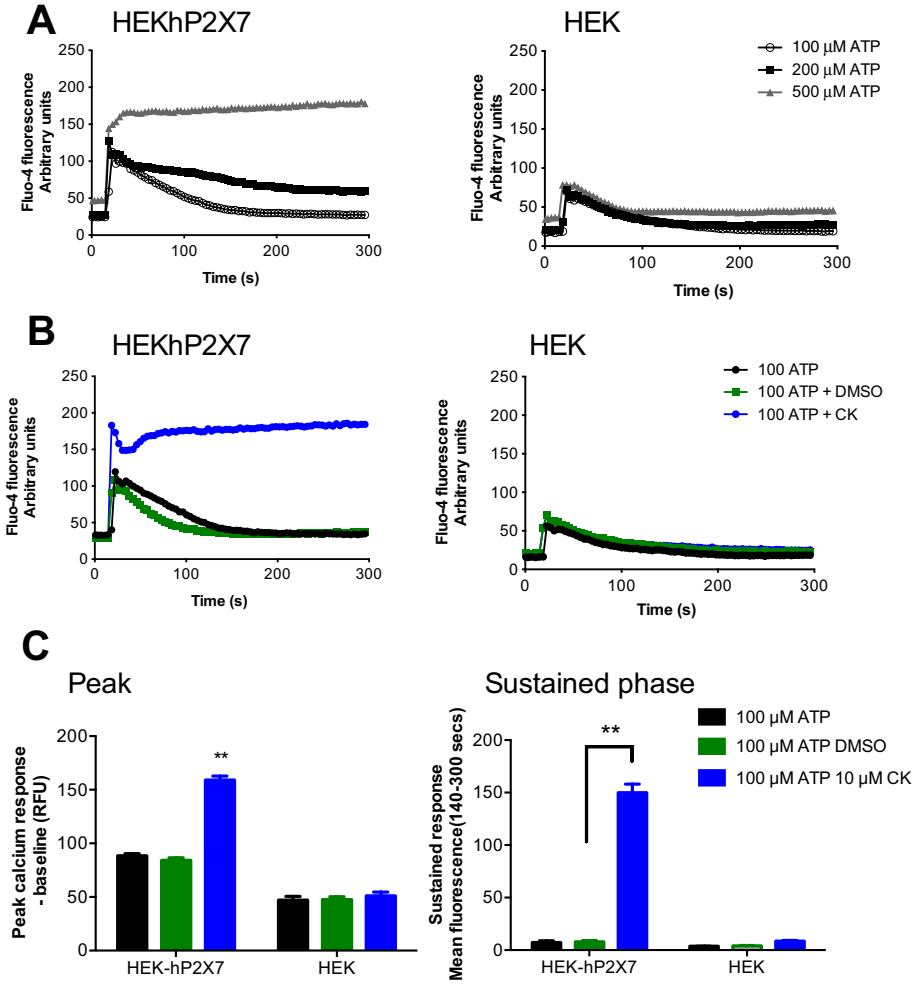

Figure 4

CK enhances the sustained calcium response associated with hP2X7 receptor activation. (A) Intracellular Ca^{2+} responses were measured in fluo-4AM loaded HEK-hP2X7 cells or untransfected HEK-293 cells. Baseline values were recorded for 15 s and then ATP was applied. Increasing concentrations of ATP (100 , 200 and 500 µM) allow a sustained Ca^{2+} response to be measured in P2X7-expressing cells. (B) HEK-hP2X7 or untransfected HEK cells were treated with 100 µM ATP, 100 µM ATP + 0.1% DMSO or 100 µM ATP + 10 µM CK and fluo-4 responses measured over time. (C) Quantitative measures of peak Ca^{2+} response (maximum–baseline) and sustained Ca^{2+} response (mean fluorescence between 140 and 300 s) in both HEK-hP2X7 and HEK cells. $^{**}P < 0.05$, significant effect of CK; one-way ANOVA with Dunnett's post-test.

Given the rapid onset and reversibility of the potentiation, it was likely that the site of interaction with P2X7 receptors was extracellular. This was confirmed by comparing the amplitude of P2X7 currents evoked by 5 s pulses of 200 µM ATP in HEK-hP2X7 cells dialysed with standard pipette solution or pipette solution containing 1 µM CK for 5 min (Figure 6A). In both groups, the amplitude of successive P2X7 currents (2 min apart) evoked by external ATP was similar; 221 ± 59 pA (n = 8) and 264 ± 48 pA (n = 3) respectively ($P > 0.05$, unpaired t-test). To confirm that the HEK-hP2X7 cells containing intracellular CK pipette solution were still responsive to extracellular CK, 500 nM CK was applied in addition to ATP for the fifth pulse (Figure 6A, open triangles) and compared with a fifth successive pulse of ATP alone in the control group. The ATP-evoked current in the presence of CK was significantly larger than after ATP alone.

To confirm that the CK potentiation of the ATP-activated current was solely mediated by P2X7 channels, we first applied 200 µM ATP for 5 s followed by a 5 s addition of CK in the continued presence of ATP (Figure 6B). This resulted in a marked potentiation of the current that was almost completely and irreversibly blocked by a subsequent addition of the selective P2X7 receptor antagonist AZ10606120 (10 µM; 93 ± 2% inhibition, n = 4 cells) in the continued presence of CK and ATP (Figure 6B).

Ginsenoside CK does not induce an immediate permeability shift to large cations

The fact that PPD ginsenosides accelerated YOPRO dye uptake in the presence of ATP might suggest that an underlying increase in the permeability of P2X7 channels to large molecular weight cations is associated with the potentiating action of ginsenosides. To investigate this possibility, cells were bathed in a solution that contained NMDG as the only extracellular cation and dialysed with standard 145 mM NaCl-containing internal solution. Under these conditions,

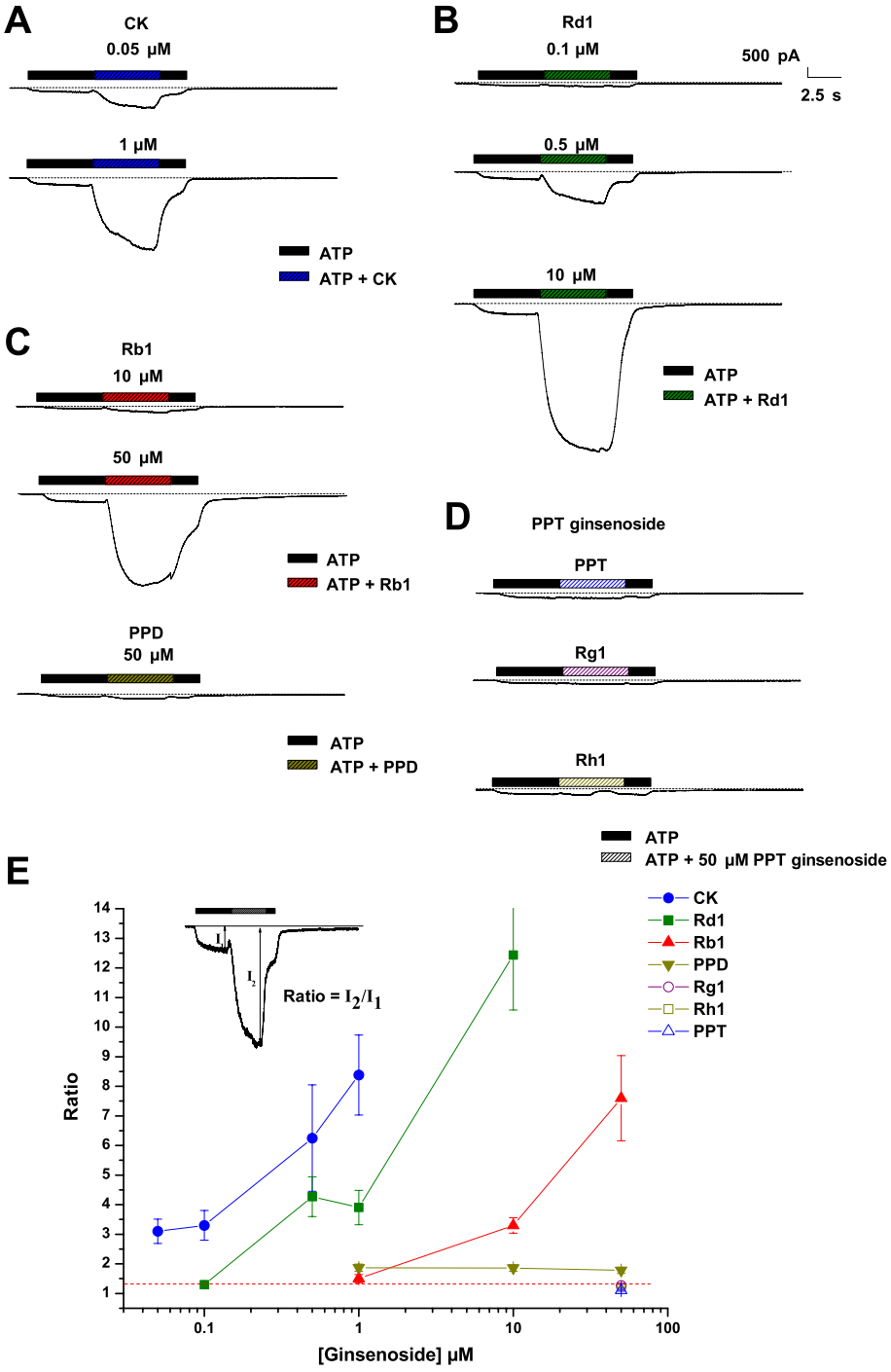

Figure 5

PPD ginsenosides rapidly and reversibly potentiate ATP-activated P2X7 currents. HEK-hP2X7 cells were voltage clamped at –60 mV and ATP was rapidly applied for 5 s, followed by a 5 s application of ginsenoside + ATP before briefly returning to ATP alone for 2 s prior to wash off. Representative traces are shown for CK (A), Rd (B), Rb1 and PPD (C), PPT, Rg1, Rh1 (D). (E) Quantification of the dose dependence of the potentiation by calculating I_2/I_1 (inset) and plotting the ratio versus [Ginsenoside] μM. Solid symbols are PPD ginsenosides and open symbols are PPT ginsenosides. PPD and all members of the PPT series (PPT, Rg1 and Rh1) do not potentiate P2X7 currents at 50 μM.

we applied 500 ms voltage ramps from –100 to +50 mV every 1 s. Fast application of ATP for 10 s evoked outward currents at all voltages positive to around –80 mV. The mean reversal potential (E_{rev}) at the end of the 10 s ATP addition (Figure 7, black bar) was –81 ± 2.1 mV (n = 4 cells) and the mean outward current amplitude at –30 mV was 1186 ± 416 pA. Solution containing ATP and 1 μM CK was then applied (Figure 7, hatched bar) which resulted in marked increase in

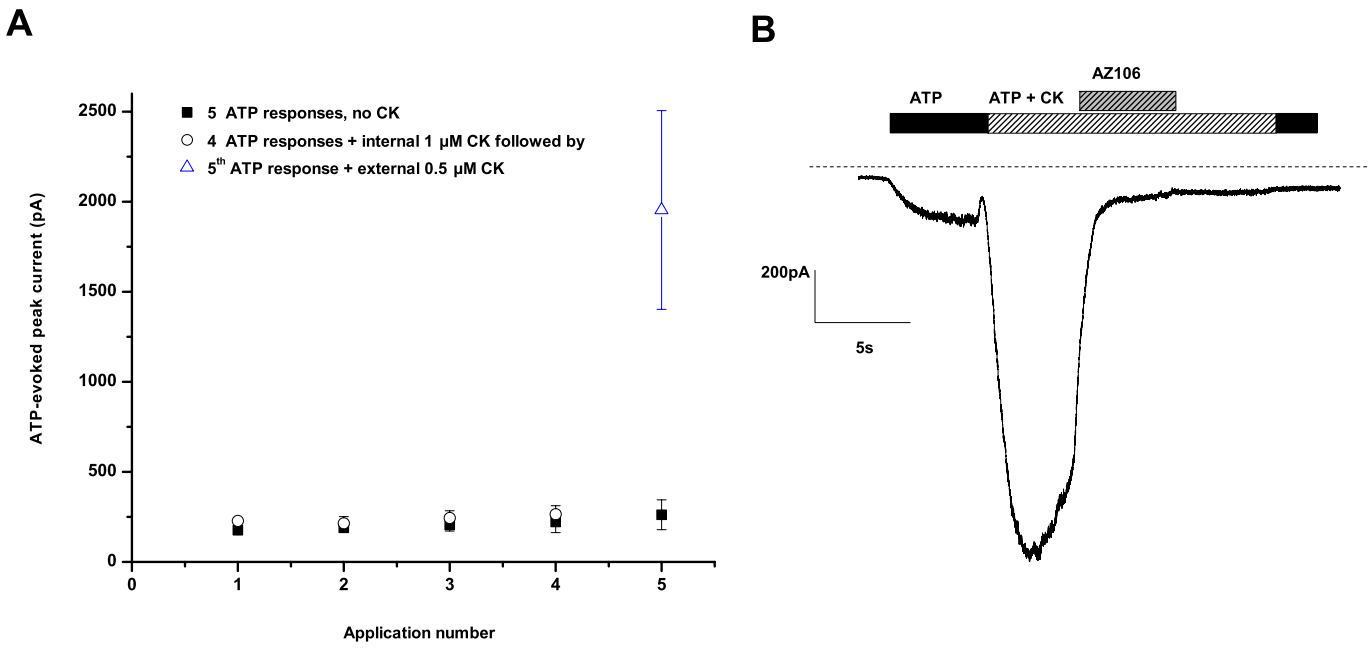

Figure 6

CK potentiation of P2X7 currents is via an extracellular site. (A) Five successive external applications of 200 μM ATP (5 s duration at 2 min intervals) produced peak inward currents of similar amplitudes in the control group. With 1 μM CK added to the pipette solution, successive external applications of 200 μM ATP produced peak inward current amplitudes similar to those recorded in the control group. Application of external CK (0.5 μM) with ATP at the fifth application in the test group of cells produced a significant potentiation of the peak inward current. (B) A representative trace showing the rapid and irreversible block of the CK-induced potentiation with the selective P2X7 receptor antagonist AZ10606120 (AZ106). ATP (200 μM) was applied for 5 s prior to the addition of ATP 200 μM plus CK 0.5 μM for a further 5 s. AZ106 (10 μM) was then applied in the continued presence of ATP and CK for 5 s prior to wash-out in ATP and CK only to assess reversibility. CK was then washed off in ATP alone for 2 s.

the amplitude of the current at –30 mV within 2–3 s of addition (mean amplitude was 3667 ± 1017 pA). Hence, under these conditions, CK potentiated the amplitude of the outward current by around threefold but this was not associated with any measurable change in permeability (E_{rev} –81.3 ± 2.4 mV). This is illustrated in Figure 7 (inset) where representative ramps in one cell are shown at the end of the first 10 s addition of ATP (ATP Ramp) and 2–3 s after the subsequent addition of ATP + 1 μM CK (ATP + CK ramp). In the continued presence of ATP + 1 μM CK, there was a progressive positive shift in E_{rev} which reached –74 ± 2 mV by the end of the 10 s addition (Figure 7). These data suggest that permeability changes do not contribute to the rapid potentiation of the P2X7-mediated current after CK addition.

Potentiation of P2X7 receptors by ginsenoside CK is not dependent on cations or membrane voltage

We further used patch-clamp recordings of HEK-hP2X7 cells to determine whether the presence of extracellular divalent cations (Ca^{2+}, Mg^{2+}) could interact with potentiation of the ATP response. The degree of potentiation of ATP-evoked inward currents was similar in standard extracellular solution (2 mM Ca^{2+}), although a higher concentration of ATP was required, 1 mM rather than 200 μM in 0.2 mM external Ca^{2+} solution (Figure 8A). There was no voltage dependence to the effect of CK as demonstrated by linear I–V relationships for ATP or ATP in the presence of CK (Figure 8B).

Ginsenoside CK potentiates responses of endogenous P2X7 receptors in mouse macrophages

We next established whether similar effects of the ginsenoside CK on endogenous P2X7 receptors could be demonstrated in mouse macrophages. We determined that CK could potentiate mouse P2X7 receptors expressed in HEK-293 cells (Supporting Information Fig. S2), thus ruling out a species-specific effect of ginsenosides. The EC_{50} value for CK potentiation of mouse P2X7 receptors was 0.45 μM (Supporting Information Fig. S2), similar to that for the CK effect on human P2X7 receptors. Using the J774 mouse macrophage cell line (Figure 9A) and primary mouse peritoneal macrophages (Figure 9B), we demonstrated enhanced sustained intracellular calcium responses to 500 μM ATP in the presence of 10 μM CK compared with 500 μM ATP alone. Furthermore, patch-clamp recordings from individual macrophages revealed a similar rapid onset of potentiation of ATP-mediated inward currents in J774 and peritoneal macrophages (Figure 9C).

Finally, we investigated whether this potentiation of ATP-induced responses could translate to a significant downstream functional effect, such as induction of apoptotic cell

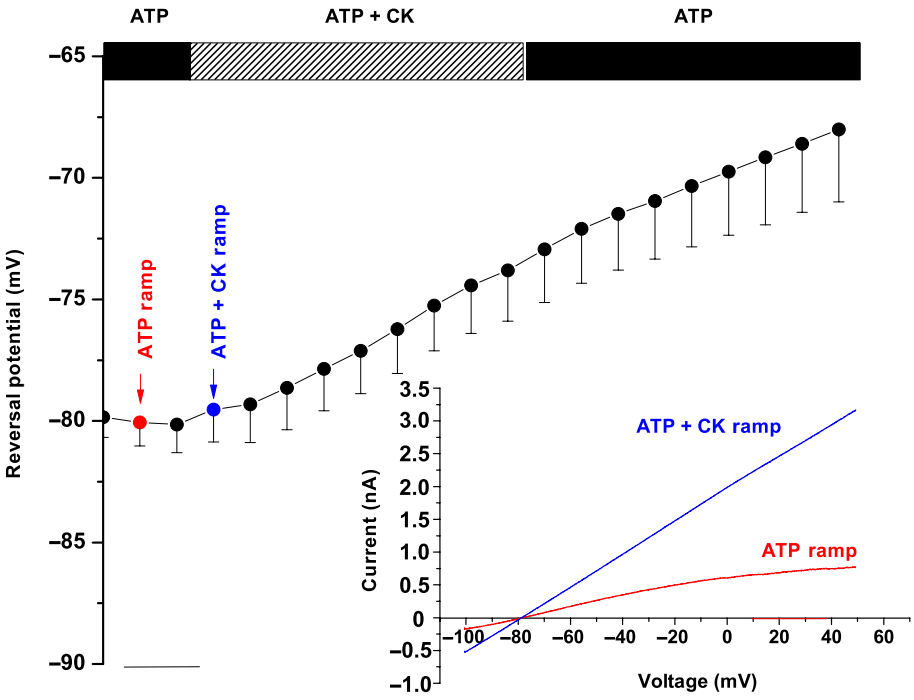

Figure 7

CK potentiation does not involve a significant change in permeability to NMDG. Cells were bathed in $NMDG^+$ external solution and a similar drug addition protocol to that in Figure 5A, using 1 μM CK (drugs applied for 10 s rather than 5 s). Voltage ramps (500 ms) were applied from −100 to +50 mV every second throughout the experiment. Typical leak-subtracted currents (inset) are shown after 10 s in 200 μM ATP (ATP) and then after 2–3 s in 200 μM ATP +1 μM CK (ATP + CK). Under both conditions, currents were outward at most voltages reflecting outward movement of Na^+ and negligible inward $NMDG^+$ movement. Even though there was a large potentiation of the ATP-evoked outward current in the presence of CK, the reversal potential did not change significantly (mean E_{rev} in ATP = −81 ± 2.1 mV vs. Mean E_{rev} in ATP + CK = −81.3 ± 2.4 mV, n = 4 cells). Maintaining the cells in ATP + CK for a further 10 s lead to a progressive positive shift in E_{rev} reflecting a progressive increase in NMDG permeability and therefore pore dilation. Re-addition of ATP alone for a further 10 s did not appear to affect the progressive positive shift in E_{rev}.

death. We used J774 macrophages and treated them with either CK alone (10 μM) or a non-lethal concentration of ATP (500 μM) for 24 h. We determined any effect on cellular viability through an 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) viability assay. Neither 10 μM CK, DMSO or 500 μM ATP alone induced any detrimental effect on cell viability (n = 4 experiments; Figure 10). In contrast, a high concentration of ATP (3 mM) for 24 h induced a significant reduction of cell viability. Treating J774 macrophages with a combination of 10 μM CK and previously non-lethal ATP (500 μM) together, now significantly reduced cell viability (Figure 10B).

Discussion

In this study, we have shown that certain ginsenosides (Rb1, Rh2, Rd and CK) markedly enhanced P2X7 receptor responses after prior activation with exogenous ATP. These effects were only manifested after previous activation of P2X7 receptors by orthostatic agonists (ATP, BzATP) and were first characterized in a HEK cell line expressing P2X7 receptors and corroborated in a macrophage cell line (J774) and mouse peritoneal macrophages. The consequences of the interaction were increased Ca^{2+} influx and a subsequent decrease in cell viability to lower concentrations of ATP. Given the widespread distribution of P2X7 channels on immune cells and the fact that effects could be observed in the submicromolar range with one of the principal metabolites of ginseng CK, it is possible that this mechanism may account for some of the reported immune modulatory actions of ginseng *in vivo*.

Because the potentiation of P2X7 receptor responses appears to be unique to glycosylated PPD ginsenosides and can be observed in the low to submicromolar range (EC_{50} of 0.45–1.08 μM), it is unlikely to be a non-specific interaction resulting from changes in membrane fluidity as has been suggested for certain ginsenoside actions reflecting the amphipathic nature of these steroid-like saponins (Attele *et al.*, 1999). In addition, the site of action is likely to be extracellular and may involve a direct interaction with the P2X7 channel as effects were not observed with intracellular application of CK (1 μM). The potentiation was rapid in onset and was rapidly reversible. However, the kinetics of onset/reversibility were not specifically addressed in this study but are at least within 1 s based on our patch-clamp experiments using a 'fast' drug application system.

The potentiating effects of PPD ginsenosides on P2X7 receptors are novel for this family of ligand-gated ion channels but do show some similarities to reported effects of ginsenosides on $GABA_A$ (Choi *et al.*, 2003b) and glycine currents (Noh *et al.*, 2003) in *Xenopus* oocytes. Although the

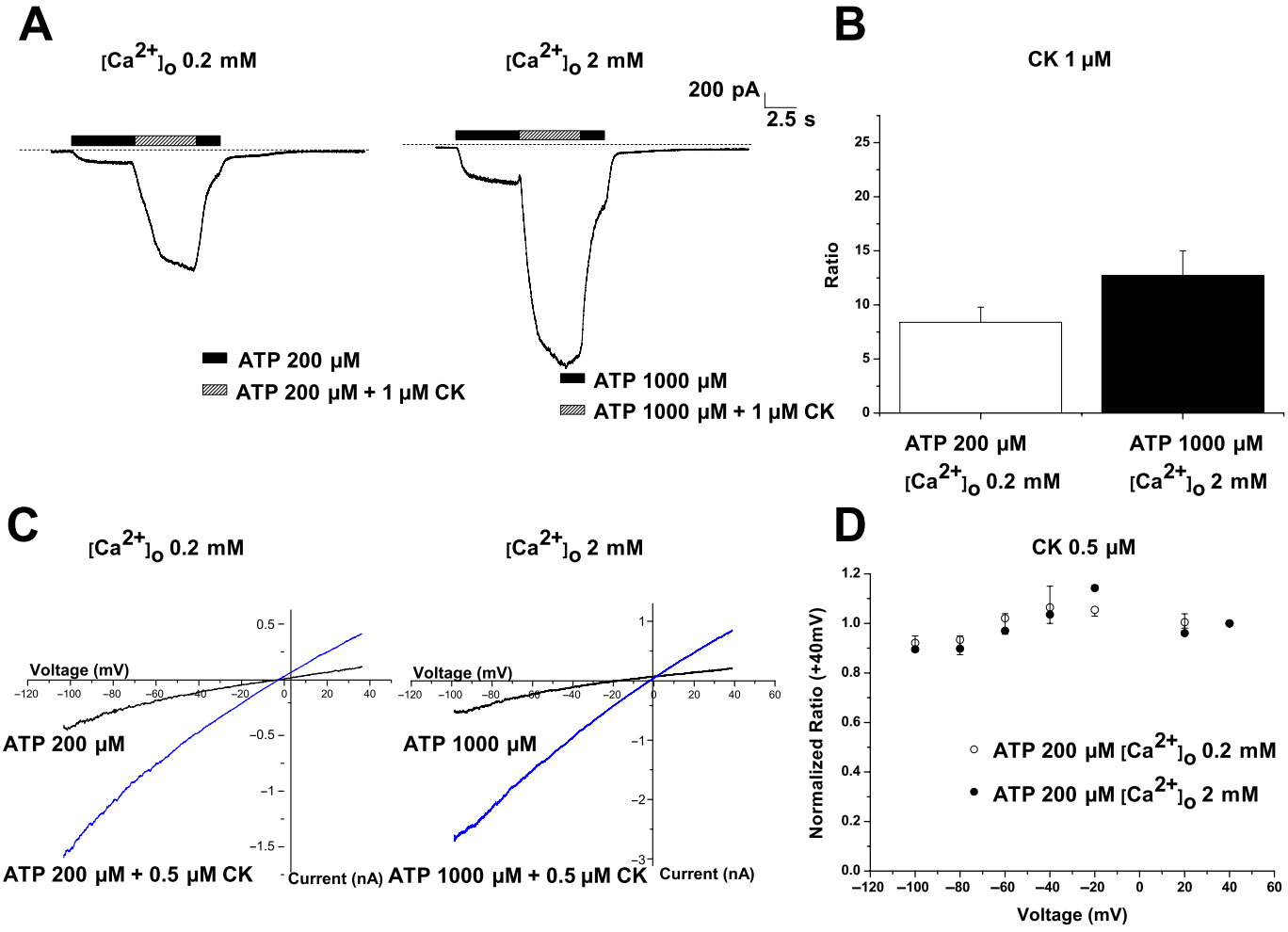

Figure 8

Ginsenoside CK potentiation of ATP-evoked P2X7 currents is observed at physiological extracellular [Ca^{2+}] and is voltage independent. (A) Using the same drug application protocol as in Figure 5, CK (1 µM) potentiated ATP (1000 µM) P2X7 currents in 2 mM extracellular Ca^{2+} to a similar extent as those mediated by ATP (200 µM) in 0.2 mM extracellular Ca^{2+}. Representative traces in 0.2 mM (left trace) and 2 mM extracellular Ca^{2+} (right trace) are shown. (B) This potentiation is quantified by calculating the mean ratio, using the method described in Figure 5E (n = 6 and 15 for P2X7 currents recorded in 0.2 mM and 2 mM extracellular Ca^{2+} respectively). (C) Leak-subtracted ramp currents were obtained after 10s in ATP only and after 10s subsequent addition of ATP and 0.5 µM CK and plotted against voltage. Ramp current /voltage relationships are shown for typical cells in the presence of either 0.2 mM (left graph) or 2 mM extracellular Ca^{2+}. Voltage ramps (1s duration) were continuously applied from –100 mV to +40 mV (every 1.25 seconds), from a holding potential of -60 mV. (D) From these ramp–current/voltage relationships, the ratio of current evoked after ATP + 0.5 µM CK and ATP alone was calculated at –100 mV and then at 20 mV increments up to +40 mV. For each cell, the ratio was normalized to the ratio at +40 mV and the mean was calculated. Data are shown for two conditions: 200 µM ATP in 0.2 mM extracellular Ca^{2+} and 1000 µM ATP in 2 mM extracellular Ca^{2+} (n = 4).

concentrations of ginsenosides necessary for the observed effects were much higher on GABA and glycine channels (around 50 µM), these studies also indicated that PPD ginsenosides were more potent than PPT ginsenosides. Such studies of effects of ginsenosides on ion channels have been extensively reviewed by Nah (2014). One PPD ginsenoside, Rg3, was additionally found to directly activate GABA channels containing the γ2 subunit (Lee *et al.*, 2013). In our study, we demonstrate that the aglycone compounds PPD and PPT were ineffective at potentiating P2X7 receptors which points to an absolute requirement of at least one sugar residue in PPD ginsenosides. Interestingly, both PPD and PPT aglycones have been shown to inhibit rather than potentiate $GABA_A$ currents (Lee *et al.*, 2012). However, we did not observe any inhibitory effects on P2X7 receptor responses with any ginsenoside compound. Further investigations are required to determine if there are any shared structural features between the ginsenoside binding sites on P2X7 and $GABA_A$ channels. This may also apply to HERG (K_v11.1) channels, as PPD ginsenosides were typically more effective than PPT ginsenosides in potentiating tail currents and PPT/PPD aglycones were ineffective (Choi *et al.*, 2011).

Glycosylated PPD ginsenosides, such as the principal ginseng metabolite CK, are novel potent positive allosteric modulators of human P2X7 receptors. Several other positive modulators of P2X7 receptors have been described including

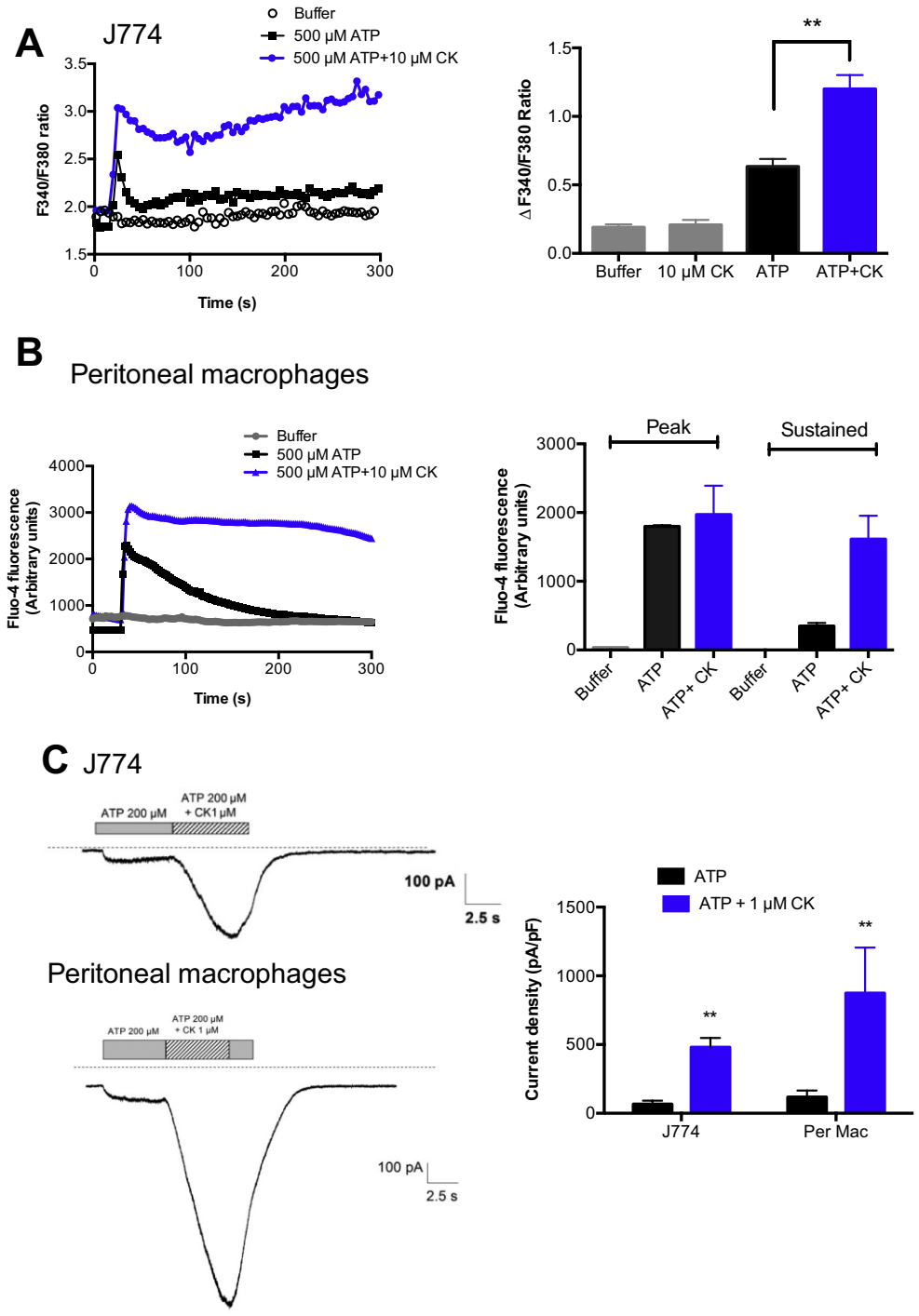

Figure 9

CK potentiates ATP-induced calcium responses and inward currents in J774 macrophages and mouse peritoneal macrophages. (A) Intracellular Ca^{2+} responses were measured in Fura-2AM loaded J774 macrophages. Baseline values were taken for 15 s and then ATP (500 μM) was applied with or without 10 μM CK. Bar chart indicates mean change in F340/F380 ratio, n = 6–11 wells from 2 independent experiments. **indicates $P < 0.05$ from one-way ANOVA with Tukey's post-test. (B) Mouse peritoneal macrophages on 12 mm glass coverslips were loaded with fluo-4AM and intracellular Ca^{2+} responses measured using a CCD camera mounted on a Nikon Eclipse Ti-U microscope. ATP (500 μM) was applied with or without 10 μM CK. Quantitative measures of peak Ca^{2+} response (maximum–baseline) and sustained Ca^{2+} response (mean fluorescence between 140 and 300 s) in peritoneal macrophages are shown in the bar chart. (C) Whole-cell patch-clamp recordings of J774 macrophages (upper) and peritoneal macrophages (lower) showing rapid potentiation of ATP-induced inward currents in the presence of 1 μM CK. In the bar graph, mean (± SEM) current densities from five to six cells are shown. **$P < 0.05$, significantly different from ATP alone; two-tailed *t*-test.

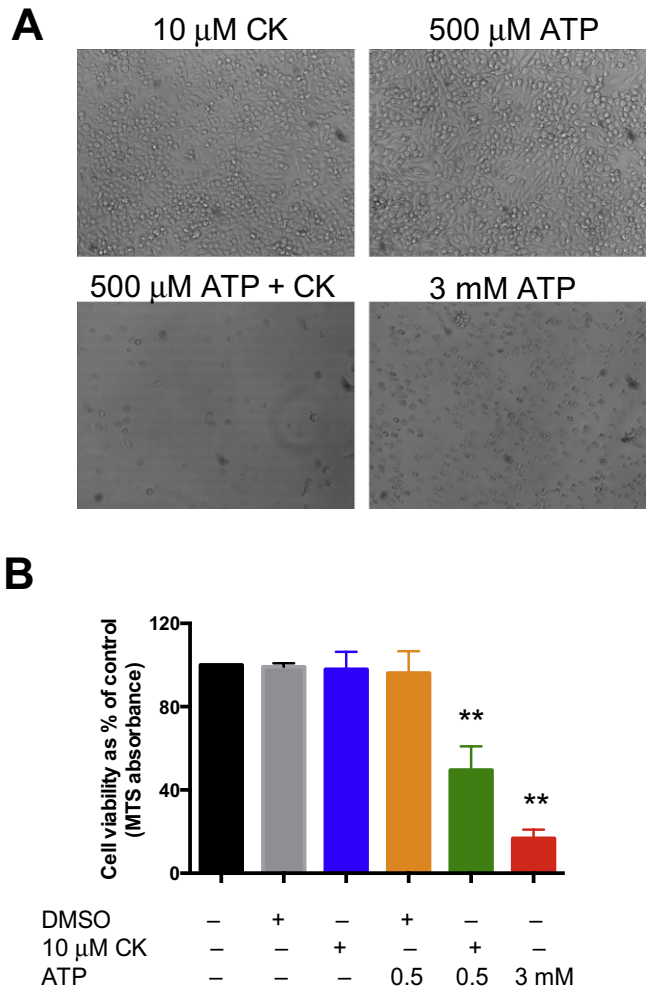

Figure 10

CK enhances the ability of ATP to cause cell death in J774 mouse macrophages. J774 cells were plated at 5×10^4 cells per well in triplicate in 96-well plates and treated with 0.1% DMSO (control), 10 µM CK, 500 µM ATP + 0.1% DMSO, 500 µM ATP + 10 µM CK or 3 mM ATP for 24 h. CellTiter Aqueous ONE solution was added for the last 4 h of treatment and absorbance was read at 490 nm using a BMG Labtech Clariostar plate reader. (A) shows cells after 18 h of treatment and and (**B**) is a summary of three independent experiments. $^{**}P < 0.05$, significantly different from media control; one-way ANOVA with Dunnett's post-test.

clemastine, tenidap, polymixin B and ivermectin (Sanz *et al.*, 1998; Ferrari *et al.*, 2004; Norenberg *et al.*, 2011; Nörenberg *et al.*, 2012). There are some similarities between the action of clemastine and CK on P2X7 channels in that their action is rapid (<100 ms), reversible, calcium- and voltage-independent and uses an extracellular site. Similar to CK, modulators such as clemastine had no direct effects on P2X7 channels and required the presence of the agonist (Norenberg *et al.*, 2011). Although Nörenberg *et al.* reported an accelerated change in reversal potential occurring over tens of seconds, reflecting an increased rate in the permeability to $NMDG^+$ in the presence of ATP, they recognized that this cannot account for the rapid (<1 s) potentiating effect and reversibility of clemastine (Norenberg *et al.*, 2011). The most plausible explanation, which may also be applicable to PPD ginsenosides, is that such modulators increase the mean-open time of P2X7 channels. Furthermore, such a net increase in channel activation is known to accelerate pore dilation. Hence, this hypothesis may reconcile our patch data which clearly indicated that the rapid and reversible effects of ginsenosides were not associated with significant changes in permeability and our YOPRO data where ginsenosides clearly increased the rate of dye uptake.

An important consequence of the PPD ginsenoside action on P2X7 channels is enhanced sustained Ca^{2+} influx in both macrophages and HEK-hP2X7 cells. The use of CK as a positive allosteric modulator of P2X7 receptors reduces the concentration of ATP required to generate a sustained Ca^{2+} response (Figures 4 and 8). Many downstream consequences of P2X7 receptor activation depend on sustained Ca^{2+} signalling (Bartlett *et al.*, 2014). With regard to cell viability, brief additions of high concentrations of ATP (<5 min, > 1 mM) can lead to a transient 'pseudoapoptosis' that does not lead to cell death (Mackenzie *et al.*, 2005) or a delayed cell death occurring after a number of hours (Hanley *et al.*, 2012). Higher concentrations of ATP and/or prolonged applications on the other hand lead to cell death within minutes because of massive Ca^{2+} influx (Mackenzie *et al.*, 2005). In contrast, lower concentrations of ATP (<1 mM) can have the opposite effect, stimulating proliferation and prolonging cell survival (Adinolfi *et al.*, 2005a). Consistent with this, we have shown that enhancing Ca^{2+} influx via P2X7 receptors in macrophages through the use of CK can effectively convert a sublethal dose of 500 µM ATP into a lethal concentration as measured by a significant decrease in cell viability after 24 h (Figure 10). However, given the fact that the timing and extent of Ca^{2+} influx via P2X7 receptors can lead to different functional outcomes, further studies are warranted with different ATP/PPD ginsenoside combinations and examination of other parameters in addition to cell viability.

n conclusion, the present study identifies selected ginsenosides as novel positive allosteric modulators of P2X7 channels. Our findings together suggest that the modulation of P2X7 receptors may account for some of the reported immune modulatory actions of protopanaxdiol ginsenosides *in vivo*.

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Author contributions

R. H., C. O. S., K. D. and L. S. performed the research. L. S., R. H., J. C. M., C. C. X. and J-M. Y. designed the study.

C. C. X. and J-M. Y. contributed essential reagents. L. S., C. O. S. and R. H. analysed the data. R. H., L. S. and C. C. X. wrote the manuscript.

Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Ginsenosides have no direct effect on rate of dye uptake responses in HEK-hP2X7 cells. Dye uptake responses were measured at 37°C using YOPRO-1 (2 μM) as the membrane impermeant dye. Relative fluorescence units (RFU) were measured following excitation at 490 nm and emission recorded at 520 nm using a fluorescent plate reader (Flexstation III). HEK-hP2X7 cells were treated with ginsenosides in a low divalent buffer at 37°C. ATP (200 μM) was used to elicit a P2X7 receptor response as a positive control. The mean of five individual wells is plotted.

Figure S2 CK ginsenoside potentiates ATP-induced responses at the mouse P2X7 receptor. (A) ATP-induced dye uptake was measured at 37°C using YOPRO-1 (2 μM) as the membrane impermeant dye. Relative fluorescence units (RFU) were measured following excitation at 490 nm and emission recorded at 520 nm using a fluorescent plate reader (Flexstation III). HEK293 cells stably expressing mouse P2X7 receptors were pretreated with 10 μM CK in a low divalent buffer for 10 min at 37°C. ATP (500 μM) was then injected to elicit a response of P2X7 receptors. The mean of 6–10 individual wells is plotted from three independent experiments. (B) A concentration-response curve for CK potentiation on mouse P2X7 receptors. Data are mean of six wells for each concentration.